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The Effects of Growth Hormone and Thyroxine Treatment on the Insulin Signaling of Female Ames Dwarf Mouse Skeletal Muscle Tissue

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THE EFFECTS OF GROWTH HORMONE AND THYROXINE TREATMENT ON THE INSULIN SIGNALING OF FEMALE AMES DWARF MOUSE SKELETAL MUSCLE TISSUE

by

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ABSTRACT

Ames dwarf (df/df) mice are deficient in anterior pituitary hormones: growth hormone (GH), thyroid stimulating hormone (TSH), and prolactin (PRL) due to a spontaneous, homozygous mutation of *prop1*^{df} gene. These dwarf mice exhibit characteristics such as delayed growth and development coupled with delayed aging, increased lifespan, overall increased insulin sensitivity, as well as resistance to certain diseases and cancers. The mutant mice possess low blood glucose, low serum insulin, and lower body temperature. Their enhanced longevity (about 40-60% longer lifespan than normal mice) is associated with their GH deficiency and disruption in the somatotropic axis (GH/IGF-1 hormonal pathway) as well as increased insulin sensitivity, which is supported by other mutant mouse models for longevity like Snell dwarfs and growth hormone receptor knock-out (GHRKO) mice. When young male Ames dwarf mice were treated with GH replacement therapy, they showed increased body growth to nearly match the normal mouse phenotype. In conjunction to an increase in physical growth, however, GH treatment also decreases the longevity and insulin sensitivity that are characteristic of these mice to levels seen in normal mice. Because of the lack of TSH, they also have undetectable levels of Thyroxine (T4). While T4 treatment didn't increase bodyweight of dwarfs to the same extent as GH treatment, the T4 treated mice retained their enhanced lifespan. Although df/df mice have enhanced whole-body insulin sensitivity, the male skeletal muscle was previously shown to be less responsive to insulin than their liver. In our study we analyzed the insulin signaling pathway in skeletal muscle from female mice after treatment with GH or GH combined with T4. Gene expression and protein expression were investigated in the skeletal muscle of female Ames dwarf mice that were treated with GH or GH and T4 therapy. Real Time Polymerase Chain Reaction

(RT-PCR) was used to analyze the expression of mRNA involved with insulin and GH signaling, while western blots were used to analyze protein expression. This project found that female Ames skeletal muscle didn't respond to GH treatment to the same extent as males, and that GH and T4 treatment tends to neutralize the effects seen in GH-only treatment.

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LIST OF ABBREVIATIONS

CR = Calorie Restriction

Df/df = Ames dwarf (homozygous mutant of *prop-1* gene locus)

GH = Growth Hormone

GH + T4 = Growth Hormone and Thyroxine

GHR = Growth Hormone Receptor

GLUT4 = Glucose Transporter 4

GTT = Glucose Tolerance Test

IGF-1 = Insulin-like Growth Factor - 1

IR = Insulin Receptor

IRS-1 = Insulin Receptor Substrate – 1

ITT = Insulin Tolerance Test

NF H_2O = Nuclease-Free Water

PGC-1α = Peroxisome proliferator activated receptor Gamma Coactivator - 1 alpha

PPAR-γ = Peroxisome Proliferator Activated Receptor - gamma

RT-PCR = Real Time Polymerase Chain Reaction

T4 = Thyroxine

TPER = Tissue Protein Extraction Reagent

TSH = Thyroid Stimulating Hormone

WT = wild-type (normal)

INTRODUCTION

The 5.8 kDa peptide hormone, insulin, has gained major attention in recent years and is practically a household term. It is an anabolic hormone secreted by β-cells of the pancreas and signals insulin sensitive tissues, such as the liver, adipose tissue, and skeletal muscle, to uptake glucose from plasma. Insulin and its signaling components are important for regulating glucose metabolism. Insulin is a pioneering protein, as it was the first protein to be sequenced (earning Frederick Sanger the Nobel Prize), the first peptide drug, the first recombinant drug, and the first hormone gene cloned [1-3]. It is the most commonly prescribed peptide drug, and used to help with glycemic control in patients with Diabetes Mellitus [4].

The metabolic disorder, Diabetes Mellitus (DM), is reaching epidemic proportions in developed nations like the United States of America, growing even in children and adolescent populations [5-7]. In the US, more than 8% of the population or 25.8 million people have diabetes as of 2011 [8]. Furthermore, the estimated total economic cost of diabetes was approximately $218 billion in 2007 [8]. Type 2 DM, formerly known as Non Insulin Dependent Diabetes Mellitus, is usually associated with co-morbidities, such as obesity or metabolic syndrome, and is the leading cause of kidney failure [7, 8]. Ninety percent of diabetics are afflicted with the Type 2 form of the disease, which is characterized by elevated blood glucose (hyperglycemia) and elevated blood lipids (hyperlipidemia) in the fasted and fed state, temporary hypersecretion of insulin, and higher insulin resistance [9, 10]. Abnormal glucose metabolism, including hyperglycemia and glucose intolerance, is associated with increased cancer risk [11].

Incidence of insulin resistance and glucose intolerance generally increases in accordance with age, beginning in the third or fourth decade, as approximately 27% of people over 65 years old are diabetic [8, 12]. Insulin signaling has been shown to have important effects on longevity and aging, as some selective alterations in this pathway can lead to increases in lifespan in several organisms, such as mice [13]. In support of this, interventions such as 30% calorie restriction (CR) has been shown to improve insulin sensitivity, delay aging, and prolong lifespan in laboratory rodents while reducing growth, body weight, and thyroid hormones [14, 15]. CR also delays onset of age associated diseases, including cancer [15].

Ames dwarf mice (df/df), have a homozygous recessive *prophet of pit-1* ($prop1^{df}$) mutation, which leads to an underdeveloped adenohypophysis (anterior pituitary). This impaired development of the pituitary gland leads to undetectable levels of circulating growth hormone (GH), prolactin (PRL), and thyroid stimulating hormone (TSH); this in turn causes suppression of circulating insulin-like growth factor 1 (IGF-1) and L-thyroxine (T_4), as well as plasma insulin and glucose [16, 17]. Meanwhile, Ames dwarf mice exhibit an increased resistance to some cancers and delayed development of malignant neoplasms [18, 19]. The GH and IGF-1 deficiencies in df/df mice may be responsible for the delayed development of fatal neoplastic lesions or tumors [18]. The df/df mouse is used as a model for research of slowed aging and extended longevity (live approximately 50% longer than normal littermates) while displaying increased insulin sensitivity in the liver yet reduced insulin response in skeletal muscle at the same signaling steps [20, 21]. “This reduced insulin response in skeletal muscle may be important for control of glucose homeostasis in these animals and have implications in their extended longevity [21].” In addition, the increased life extension of Ames dwarf feature similar

characteristics to normal mice in a CR regimen (like increased insulin sensitivity); however, CR is not an Ames emulation [18, 22]. Interestingly, when CR is utilized in conjunction with Ames dwarf mice, there is further extension of lifespan in the already long lived mutants [14].

Overall, Ames dwarf mice have undetectable GH levels and increased responsiveness in terms of insulin signaling, which has been associated with their longevity [23]. GH is an important modulator of insulin sensitivity, as excess concentrations of GH are associated with hyperinsulinemia, a decrease in insulin receptor (IR) levels, and a decrease in the activity of IR kinase [24-26]. GH doesn't directly interact with the insulin receptor (IR), but its diabetogenic effects possibly result from signaling cross-talk between GH and insulin pathways as several post-receptor events are shared between the two. The membrane associated insulin receptor substrate (IRS) proteins of the insulin pathway seems to be a focal point where the signaling pathways can intersect (see Supplement 1 in Appendix). Both use the PI3-K / Akt pathway as part of their downstream signaling cascades. Furthermore, GH has been described to have acute effects on carbohydrate metabolism that are insulin-like, including translocation of GLUT4 to the plasma membrane, yet anti-insulin chronic effects [24]. Insulin sensitive tissues include the liver, adipose tissue, and skeletal muscle tissue. Skeletal muscle tissue is the largest insulin-sensitive organ in the body, so insulin resistance in this striated tissue will have a major impact on whole-body glucose homeostasis [27]. There are a few suggestions on how GH affects insulin signaling in skeletal muscle. As stated by Dr. Dominici: *"In skeletal muscle, GH-induced insulin resistance might involve an increase in the amount of the p85 subunit of PI3K that plays a negative role in insulin signaling. GH also reduces insulin sensitivity by enhancing events that negatively modulate insulin signaling such as stimulation of serine phosphorylation of IRS-1,*

which prevents its recruitment to the IR [24].” In other words, GH signaling may negatively regulate the insulin signaling cascade by affecting the quantity or activation of downstream proteins shared between the two.

Because they also lack the anterior pituitary hormone, TSH, Ames dwarf mice are also severely deficient in thyroid hormones. Thyroid hormones regulate development, thermogenesis, and metabolic homeostasis of most cells. After stimulation by TSH, the thyroid gland mostly secretes thyroxine (T4) into the bloodstream to circulate until it reaches target tissues. At these target tissues, T4 is converted into the more potent triiodothyronine (T3) to exert actions on regulating energy metabolism. The scarcity of T4 in Ames dwarf mice leads to a lower body temperature compared to normal mice, which may tie in to the rate of living theory of aging since body temperature can be indicative of metabolic rate; this theory suggests that an organism’s lifespan is inversely correlated with its metabolic rate [16, 28]. In a previous study using the same animal groups as our project, combination treatment of GH and T4 in Ames dwarf mice increased their bodyweight to nearly match normal control mice at 84% [29]. As demonstrated in another study, early life treatment of just T4 slightly increases bodyweight in female dwarfs and doesn’t reduce longevity, unlike early life GH treatment [30]. We hypothesized that the combination of GH and T4 therapy to young, developing Ames dwarf mice will alter genetic expression to reduce the mutants’ insulin sensitivity to match normal mice more closely than GH treatment alone.

In this project, we examined the effects of GH treatment and GH + T4 combination treatment on insulin signaling in female Ames dwarf mouse skeletal muscle tissue. This study

was based on previous experiments [26, 29]. It has been reported that GH treatment in Ames dwarf mice decreases insulin sensitivity [26]. This study was performed to determine if the alterations in whole body insulin sensitivity after GH and T4 treatment in Ames dwarf mice are due to genetic and/or protein changes of the insulin signaling pathway in skeletal muscle by hormonal therapy. We can help determine which components of the insulin signaling pathway are affected by growth hormone. This study is significant, as it will help elucidate the effects that GH and T4 may play on insulin signaling and therefore improve understanding of insulin resistance, which is a characteristic of aging as well as major pathologies such as type 2 diabetes and metabolic syndrome.

MATERIALS AND METHODS

Animals

In a breeding colony, normal heterozygous (N/df) females were mated with homozygous (df/df) mutant males to produce the Ames Dwarf and N/df mice. The mice were housed under light- and temperature- controlled conditions, with a 12-hour light and 12-hour dark cycle and the temperature maintained at 20-23°C. Nutritionally balanced food was provided ad libitum (Rodent Laboratory Chow 5001: 23.4% protein, 4.5% fat, 5.8% crude fiber; LabDiet PMI Feeds, Inc., St Louis, MO). The animal study was completed at Southern Illinois University in Springfield Illinois following SIU Laboratory Animal Care Committee approval. [26, 29]

This experiment analyzed four different groups of female mice: a normal control group of N/df treated with 0.9% saline, a mutant control group of Ames Dwarfs treated with 0.9% saline, Ames Dwarfs treated with porcine-GH, and Ames Dwarfs treated with GH and T4. There are 8-10 animals belonging to each group. GH treatment started at 2 weeks of age and lasted for 6 weeks, ending when the mice were 8 weeks of age. After this 6 week treatment period, the mice were fasted overnight for insulin stimulation and then sacrificed by cervical dislocation 12 hours later. Half of the animals in each group (4-5 mice per group) were injected with porcine insulin (Sigma, St. Louis, MO) at dose of 10IU/kg bodyweight via the inferior vena cava before being sacrificed. Fasting blood glucose levels were also measured before sacrifice from blood collected through the tail vain using the OneTouch Ultra glucose meter (Life Scan, Inc. Milpitas, CA). The other half was treated with saline instead of insulin before cervical dislocation as a control.

Samples tissues were harvested and frozen on dry ice immediately to be stored at -80°C until analysis.

Treatment

For preparation of the GH, porcine-GH (Alpharma, Victoria, Australia) was dissolved in 0.1M $NaHCO_3$ for stability, then 0.9% saline was added to make a single injection concentration of 21µg/50µL for the average animal bodyweight of 7g (3µg/g of bodyweight). GH injections were performed twice a day on weekdays with each injection administering half the daily dose (~3µg/g); the first injection was ~9am and the second was ~4pm (~6µg/g/day total GH treatment). On weekends, only one injection was performed with the full daily dosage (~6µg/g/day).

For the preparation of the T4, L-thyroxine (Sigma, St Louis, MO) was placed in a 0.9% saline solution with a pH of 7.8. It was administered to the mice three times per week (Monday, Wednesday, and Friday) via subcutaneous injections (0.1µg/g body weight; 0.7 µg/50µL dose).

Preliminary experiments, based on previous studies, in the Ames dwarf colony were used to determine the dosage for treatment [26].

Methodology

Insulin Tolerance Test (ITT) was performed after 5 weeks of GH treatment to separate set of female mice that underwent the same duration and dosage of treatment. The groups of mice also included wild-type normal controls, untreated Ames dwarf controls, and GH treated Ames dwarfs. For the ITT procedure, food was removed on the morning of the test, 2 hours before the first glucose measurement. A Glucometer ONE Touch Ultra (Life Scan, Inc.) was used to measure basal glucose in blood from the tip of the tail. The mice were then injected with 0.75IU/kg of bodyweight of porcine insulin (Sigma, St. Louis, MO); blood glucose was measured by Glucometer 15, 30, and 60 minutes after insulin stimulation [26]. Three days after ITT, Glucose Tolerance Test (GTT) was performed. Mice were fasted overnight and in the following morning after initial blood glucose measurement, animals were injected with 2 grams per kilogram of bodyweight of glucose solution intraperitoneally. Following the injection, blood glucose was measured at: 15 min, 30 min, 60 min, and 120 min using the Glucometer, same as in the ITT procedure [26].

Gene and protein analysis was performed directly from the skeletal muscle tissue samples harvested from the animals stated above. Real Time Polymerase Chain Reaction (RT-PCR) was used to analyze expression of the following genes: IR, IRS1, IGF-1, GHR, GLUT4, Akt2, PGC-1α, PI3K, PPAR-γ, STAT1, STAT5a, and STAT5b. The Western Blot technique was used to analyze the expression of the proteins Akt and p-Akt [Ser^{473}].

Statistics

Microsoft Excel and GraphPad Prism5 software was used to calculate the raw data. RT-PCR calculation equation can be found in the *Gene Expression Analysis* section. Statistics were calculated on Prism5 via two-tailed P values for unpaired t tests. Differences between groups that generated a p value < 0.05 were considered statistically significant.

Gene Expression Analysis:

First, mRNA was extracted from the sample tissue using the Qiagen miRNeasy Mini Kit. Approximately 75mg of sample tissue was cut from each skeletal muscle sample, and then placed in a 1.5mL safe-lock tube with 1mm Zirconium oxide beads and 700µL of QIAzol Lysis Reagent. Samples were homogenized in a bullet blender at speed 10 for 2 minute periods, and then cooled on wet ice for 2 minutes. This blending and cooling was repeated 3 times per sample or until well homogenized. From here, the Quick-start Protocol included in the kit was followed as outlined by Qiagen, including the DNase digest steps. The result of using the kit was approximately 40µL of purified mRNA in nuclease free water (NF H_2O). RNA concentration was approximated using agarose gel electrophoresis as well as Nanodrop procedure with the Epoch Gen5 Plate Reader (see appendix for procedure details).

Following mRNA extraction was cDNA synthesis with the Bio-Rad kit. After the mixture of master-mix (5x iScript reaction mix and iScript reverse transcriptase) with mRNA templates (derived from the tissue samples) in PCR tubes according to protocol, the reaction mix samples

were placed into a Bio-Rad MJ Mini Personal Thermal Cycler. The machine conditions were set at 25°C for 5 min, at 42°C for 30 min, at 85°C for 5 min, then hold at 4°C when finished. This lead to cDNA samples in 20µL of NF H_2O; 40-60µL of NF H_2O was added to each sample to dilute them and narrow the range of B_2M cycle threshold (C_T) values with RT-PCR (see below). The cDNA samples were stored at 4°C until analysis (if there was a long period of time between cDNA synthesis and RT-PCR analysis, the cDNA samples were frozen at -20°C).

This cDNA was used for RT-PCR analysis. 2µL of cDNA was used per sample in a 96 well PCR plate. The forward and reverse primers for the gene of interest were prepared with SYBR Green and NF H_2O in a master mix according to Applied Biosystems protocol, then added to the 96 well plate containing the cDNA samples for a total of 20µL per well. For PCR protocol and table of primers used, see appendix.

To normalize the RT-PCR data, Beta 2 Microglobulin (B_2M) was selected as the housekeeping gene. When C_T values had a maximum range of 3 between all samples, gene analysis began. The following equation was used for calculating relative expression: $2^{A-B}/2^{C-D}$ (A = C_T number of the gene of interest in the first control sample, B = C_T number of the gene of interest in each sample, C = C_T number of B_2M in the first control sample, D = C_T number of B_2M in each sample). This lead to the first control sample having a relative expression of 1, and all other samples were calculated in relation to this first sample. The results of the normal (N/df) group were averaged, and all other results were divided by this average to obtain the fold change of expression of the genes of interest compared to this control group [29].

Protein Analysis:

Protein was extracted from the skeletal muscle tissue using Thermo Scientific's Tissue Protein Extraction Reagent (TPER) protocol. 100mg tissue was cut from the skeletal muscle sample and placed in a 1.5mL safe-lock microcentrifuge tube with 1mm Zirconium oxide beads and 300µL of TPER. Samples were well homogenized using the bullet blender at speed 10 for 2 minute periods, then cooled on wet ice for 2 minutes between each run. This blending and cooling process was repeated 3 to 4 times per sample until well homogenized. The samples were then centrifuged at 13,000 rpm for 10 minutes at 4°C to pellet the tissue/cellular debris. The supernatant was collected via micropipette, carefully avoiding any intramuscular fat that settled on the surface of the solution. The samples were frozen at -80C until ready for analysis.

The Pierce BCA Protein Assay Kit (Thermo Scientific) was used according to the microplate procedure to determine total protein concentration in each sample. The BCA assay is a copper (II) reduction colorimetric protein assay; it is similar to the Bradford Assay. 1:10 dilution was used; 2.4µL sample protein was mixed with 21.6µL of TPER for a total of 24µL per BCA sample. 10µL of each diluted BCA protein sample was pipetted per well into a 96 well cell-culture plate in duplicates; bovine serum albumin (BSA) standards were added to the plate in duplicates as well. 200µL of BCA working reagent was added to each well and the plate was incubated at 37°C for 30 min. After incubation, the plate was cooled to room temp and then absorbance was analyzed in the Epoch Gen5 Plate reader at 562nm. The protein concentrations were automatically extrapolated from a standard curve generated from set concentrations of BSA. With this protein concentration data, Microsoft Excel was used to calculate the volume of

each protein sample to be used in order to load 60μg of protein within a 14μL volume per well. 4X sample buffer (with β-mercaptoethanol in a 19:1 ratio) was added to each protein sample in a 1:3 ratio (sample buffer : sample protein + TPER).

SDS-PAGE was performed using 26 well 10% Criterion TGX Precast Gels (stored at 4°C until use). The electrophoresis and western transfer procedure specifications are included in the appendix. Pierce Reversible Protein Stain Kit for PVDF membranes (Thermo Scientific) was used as a loading control. This allows one to easily visualize all nonspecific protein bands within the membrane. Normally β-actin or β-tubulin housekeeping proteins would be used as loading controls for normalization and monitor for equal protein loading. However, probably due to the complication of smaller cell size for Ames dwarf mice, this lead to an inconsistent comparison between dwarf and normal groups even when the same amount of protein was pipetted in each well. In any case, these housekeeping proteins were not expressed uniformly, leading to potential normalization issues. These issues were overcome by staining all proteins indiscriminately and using this data to monitor relative protein loading.

Membranes were imaged with the Li-Cor Odyssey Infrared Imager and ImageStudio Version 2.1 software. Median background noise was subtracted from signal value to produce the true band intensity values. These values were used in GraphPad Prism 5 software to generate comparison graphs and for statistical analysis. Specific details and information regarding the antibodies used can be found in the appendix.

RESULTS

Bodyweight

Ames dwarf mice are significantly smaller than normal (wild-type) control mice ($p < 0.0001$). However, after either GH or GH and T4 treatment, the dwarf mice grow significantly larger and match the bodyweight of normal mice ($p < 0.0001$) (Figure 1).

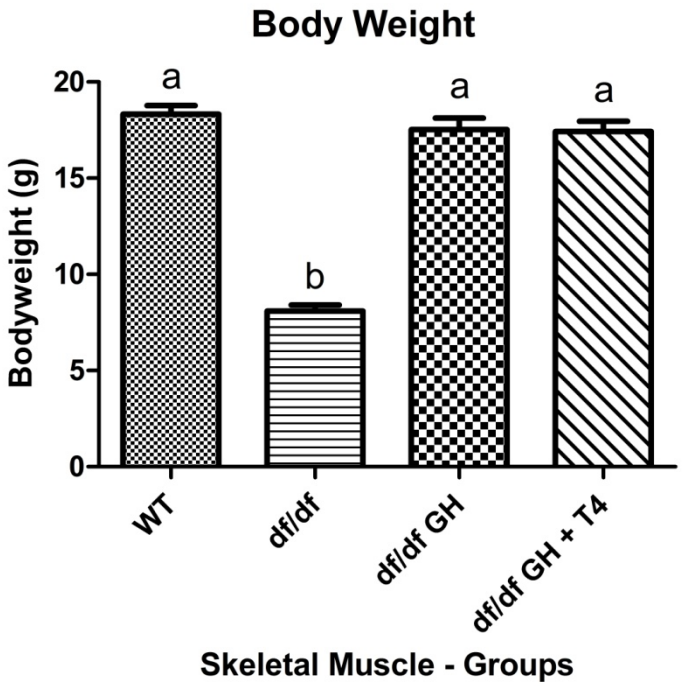


Figure 1 – Bodyweight comparison of female wild-type (WT) mice, untreated Ames dwarf mice (df/df), GH treated Ames dwarf mice (df/df GH), and Ames dwarf mice treated with both GH and T4 (df/df GH + T4). WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH + T4, n = 8. Groups that do not share a superscript are significantly different ($p < 0.05$).

Fasting Blood Glucose

GH treatment to female Ames dwarf mice increased fasting blood glucose levels compared to normal mice ($p = 0.0163$). However, GH and T4 treatment showed no significant increase above wild-type mouse fasting blood glucose levels, instead matching untreated Ames dwarfs (Figure 2).

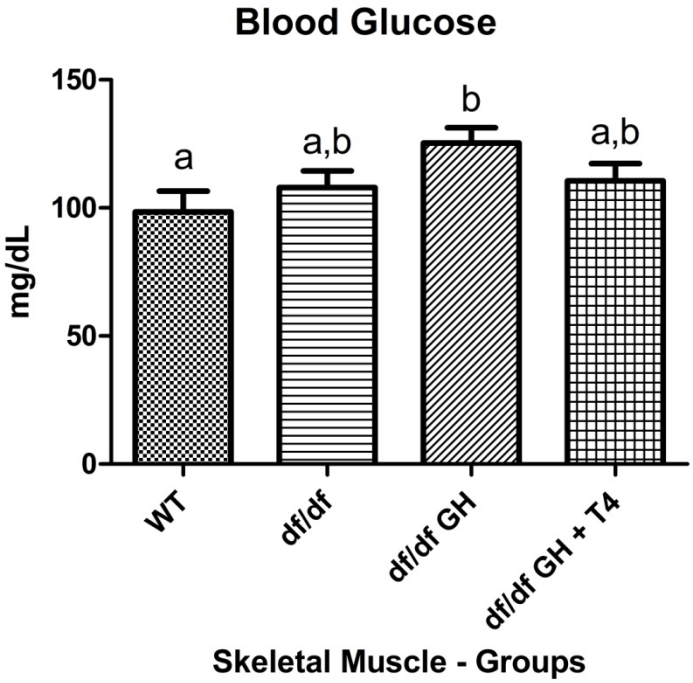


Figure 2 – Fasting blood glucose measurements in wild-type mice (WT), Ames dwarf mice (df/df), Ames dwarf mice treated with GH (df/df GH), and Ames dwarf mice treated with both GH and T4 (df/df GH + T4). WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH + T4, n = 8. Groups that do not share a superscript are significantly different ($p < 0.05$).

Insulin Tolerance Test and Glucose Tolerance Test

ITT and GTT were performed in a separate study using the same mouse models, the same treatment regimen, and the same dosages. Female Ames dwarf mice were treated with GH for 6 weeks, starting at 2 weeks of age, using the same dose as described above; then their insulin tolerance and glucose tolerance were compared to untreated wild-type mice and untreated dwarfs.

For both the ITT and GTT, GH treatment did not demonstrate a significant decrease in either whole-body insulin sensitivity or glucose tolerance when compared to untreated dwarfs, who are characterized by improved ITT and GTT (Figures 3 and 4).

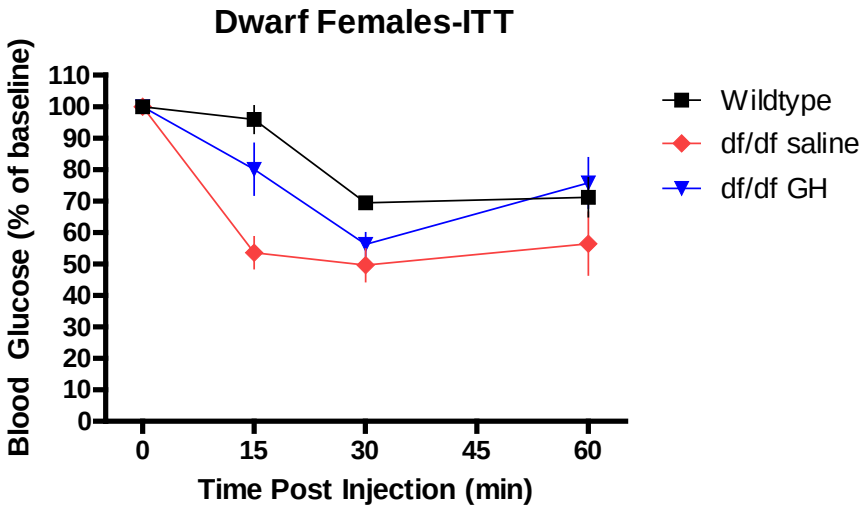


Figure 3 – Insulin Tolerance Test of GH treated female Ames dwarf mice compared to age and sex-matched normal and dwarf controls. X-axis indicates Time in minutes; Y-axis indicates percent of Blood Glucose measurements compared to baseline.

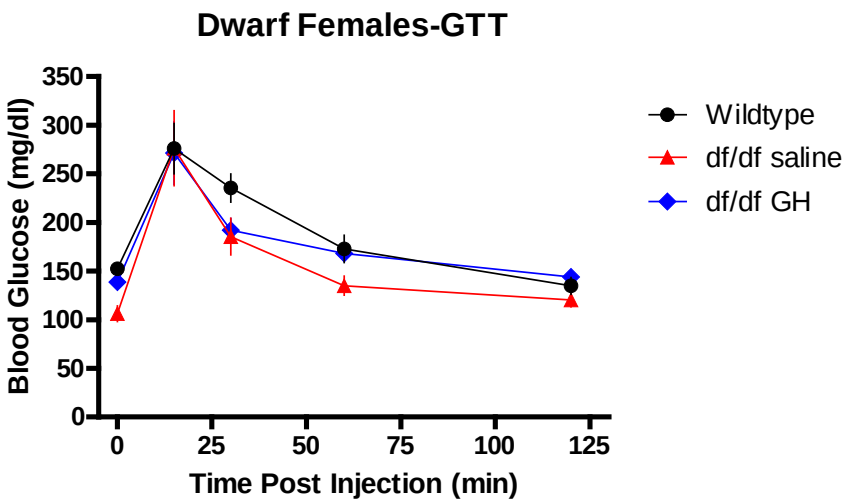


Figure 4 – Glucose Tolerance Test (GTT) of GH treated female Ames dwarf mice compared to age and sex-matched normal and dwarf controls. X-axis indicates Time in minutes; Y-axis indicates Blood Glucose in mg/dl.

mRNA Expression

Insulin receptor mRNA levels significantly decreased in mice treated with both GH and T4 (GH+T4) when compared to normal (p=0.0064), saline treated dwarfs (p=0.0255), and GH treated df/df mice (p=0.0097) (Figure 5-A). While normal and saline treated dwarfs had the same levels of IRS-1 mRNA, GH treated dwarfs showed a significant increase compared to both groups (WT vs. df/df GH p=0.0150; df/df vs. df/df GH p=0.0179). However, GH + T4 treated dwarfs showed no significant differences between any of the other groups (Figure 5-B). While saline treated dwarfs and GH treated dwarfs showed no difference in PI3-K mRNA levels with any groups, GH+T4 treated mice show about half the expression compared to normal mice

(p=0.0332) (Figure 5-C). Evident in Figure 5-D, normal mice and GH treated mice share the same relative expression of Akt2 mRNA, yet Akt2 mRNA levels decreased in GH+T4 treated dwarfs compared to both groups (WT vs. df/df GH+T4 p=0.0118; df/df GH vs. df/df GH+T4 p=0.024). Saline treated dwarfs have numerically lower levels of expression compared to normal and GH treated dwarfs, but show no statistically significant differences between any groups.

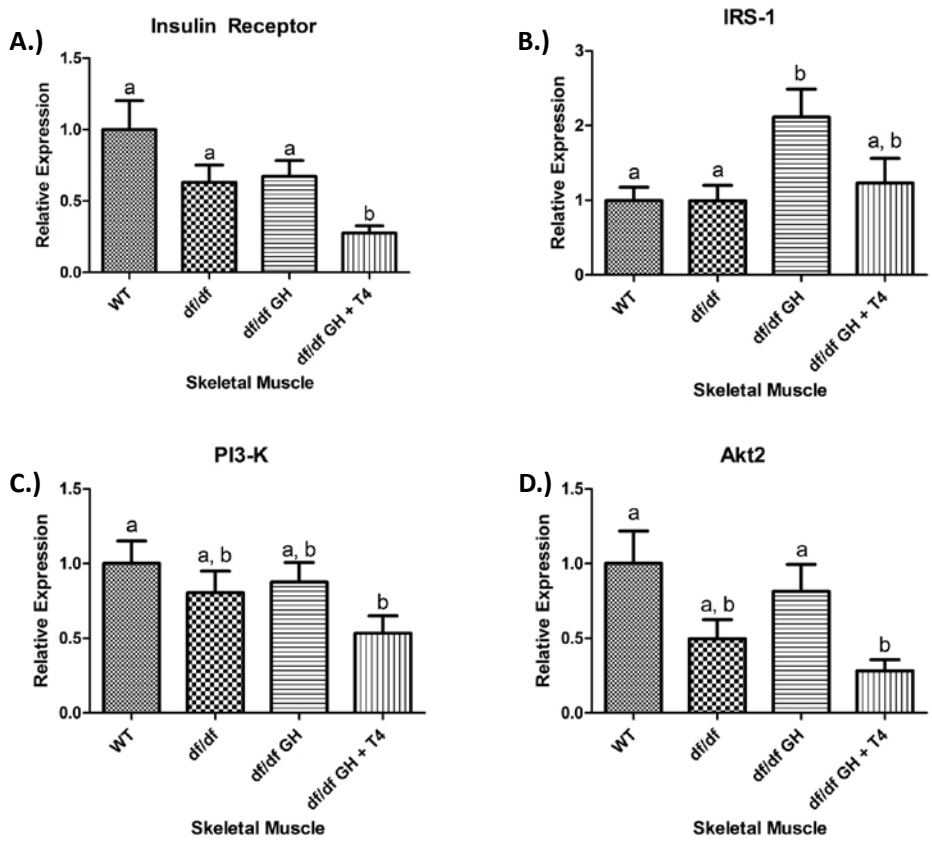


Figure 5. Relative gene expression of insulin receptor (IR), insulin receptor substrate 1 (IRS-1), PI3-K, and Akt2 mRNA in female Ames dwarf (df/df) mice after growth hormone (GH) and thyroxine (T4) treatment. X-axis represents different animal treatment groups; Y-axis represents relative mRNA expression of the gene of interest. (A) Skeletal Muscle IR mRNA in wild-type (WT), n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. (B) Skeletal Muscle IRS-1 mRNA in WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. (C) Skeletal Muscle PI3-K mRNA in WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. (D) Skeletal Muscle Akt2 mRNA in WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. Groups that do not share a superscript are different with statistical significance ($p < 0.05$).

PPAR-γ mRNA for normal and GH treated dwarfs were at equal levels, yet showed no statistical differences with any groups. However, GH+T4 treatment in dwarfs elicited a significant reduction in PPAR-γ expression compared to saline treated dwarfs ($p=0.0267$) (Figure 6-A). Although normal mice had numerically higher levels PGC-1α mRNA compared to all other groups, statistics showed no significant differences (Figure 6-B). Growth hormone receptor (GHR) expression shares a similar expression pattern to Akt2, as demonstrated in Figure 6-C. Normal and GH treated dwarfs have the same GHR mRNA levels while GH+T4 treatment suppresses expression to approximately half ($p=0.0076$ and $p=0.0232$ respectively). Again, saline treated dwarfs show no statistically significant differences with any groups. On the other hand, saline treated dwarfs showed higher expression of IGF-1 mRNA compared to normal mice ($p=0.0073$). Treatment of dwarfs with GH brought IGF-1 mRNA to substantially higher levels than both normal and saline treated dwarf groups ($p=0.0005$ and $p=0.0031$ respectively). However, the addition of T4 treatment in conjunction with GH brought levels back down to match saline treated dwarfs (WT vs. df/df GH+T4 $p=0.0066$; df/df GH vs. df/df GH+T4 $p=0.0231$) (Figure 6-D).

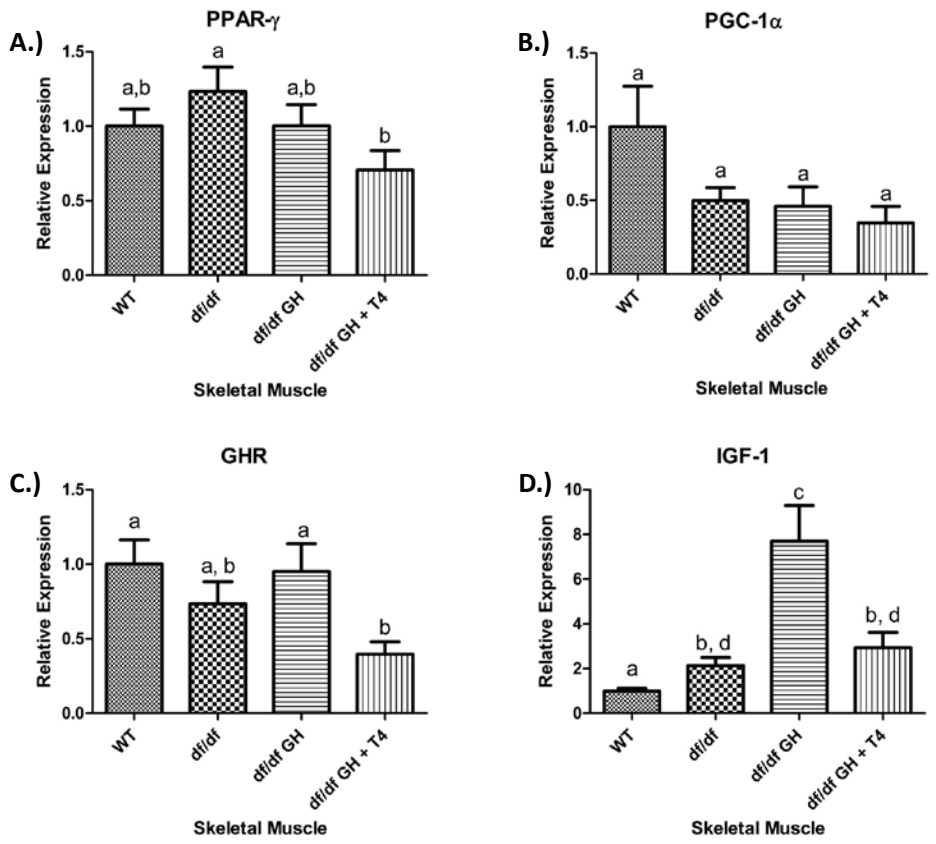


Figure 6 - Relative gene expression of Peroxisome Proliferator Activated Receptor gamma (PPAR-γ), PGC-1α, growth hormone receptor (GHR), and IGF-1 mRNA in female Ames dwarf (df/df) mice after growth hormone (GH) and thyroxine (T4) treatment. X-axis represents different animal treatment groups; Y-axis represents relative mRNA expression of the gene of interest. (A) Skeletal Muscle PPAR-γ mRNA in wild-type (WT), n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. (B) Skeletal Muscle PGC-1α mRNA in WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. (C) Skeletal Muscle GHR mRNA in WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. (D) Skeletal Muscle IGF-1 mRNA in WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. Groups that do not share a superscript are different with statistical significance ($p < 0.05$).

STAT-5a gene expression showed no differences between any groups (Figure 7-A). However, GH treatment significantly suppressed STAT-5b gene expression in dwarf mice (p=0.0376) and GH and T4 treatment suppressed expression even further (p=0.0188) (Figure 7-B). STAT-1 mRNA expression followed the same trends as Akt2 and GHR. Normal and GH treated dwarfs have the same STAT-1 gene expression, while GH+T4 treatment of dwarfs were

significantly less than both (p=0.0098 and p=0.0054 respectively); saline treated dwarfs showed no major differences with any groups (Figure 7-C). Saline treated dwarf mice had a lower mRNA expression of GLUT4 compared to normal mice (p=0.0093); GH treatment of dwarfs corrected the GLUT4 mRNA expression back to levels of normal mice, which is higher than the saline treated dwarfs (p=0.0143). However, GH and T4 treatment dropped GLUT4 gene expression back down to levels lower than normal, dwarf, and GH treated dwarf mice (p=0.0044, p=0.0432, p=0.0016 respectively) (Figure 7-D).

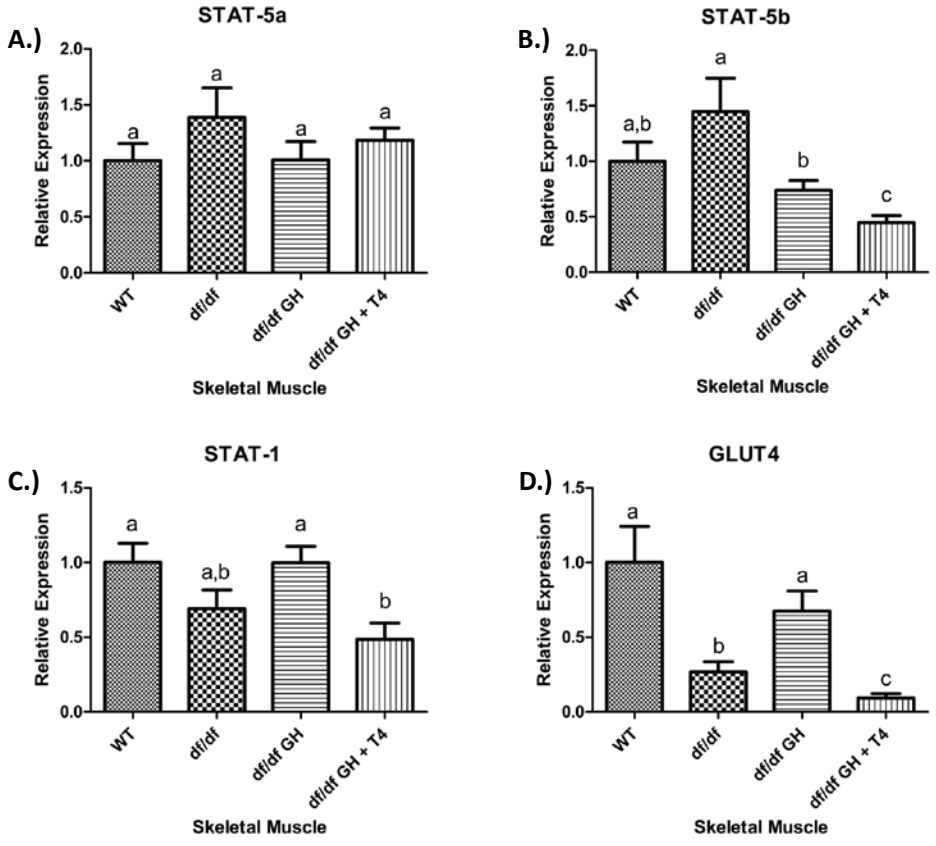


Figure 7 - Relative gene expression of STAT-5a, STAT-5b, STAT-1, and GLUT4 mRNA in female Ames dwarf (df/df) mice after growth hormone (GH) and thyroxine (T4) treatment. X-axis represents different animal treatment groups; Y-axis represents relative mRNA expression of the gene of interest. (A) Skeletal Muscle STAT-5a mRNA in wild-type (WT), n = 9; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. (B) Skeletal Muscle STAT-5b mRNA in WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. (C) Skeletal Muscle STAT-1 mRNA in WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. (D) Skeletal Muscle GLUT4 mRNA

in WT, n = 10; df/df, n = 10; df/df GH, n = 10; and df/df GH+T4, n = 8. Groups that do not share a superscript are different with statistical significance ($p < 0.05$).

Protein Expression

The protein analysis providing successful Western Blot data were total Akt (Figure 8) and p-Akt Ser473 (Figure 9). Preliminary Western Blot runs of p-Akt [s473] showed no signal and no expression levels in saline stimulated mice. Because of this, only insulin stimulated mice were included in the following blot to increase the number of mice per group. During statistical analysis of the p-Akt [s473] blot, a couple of the outlier sample values were omitted from the data set; preliminary western blot data indicated an incomplete insulin stimulation of these omitted mice, providing artificially low phosphorylation levels and skewing the data. By omitting this skewed data, the standard error decreased. As seen in Figure 9, untreated and GH treated dwarf mice had lowered total Akt expression compared to normal mice ($p < 0.01$).

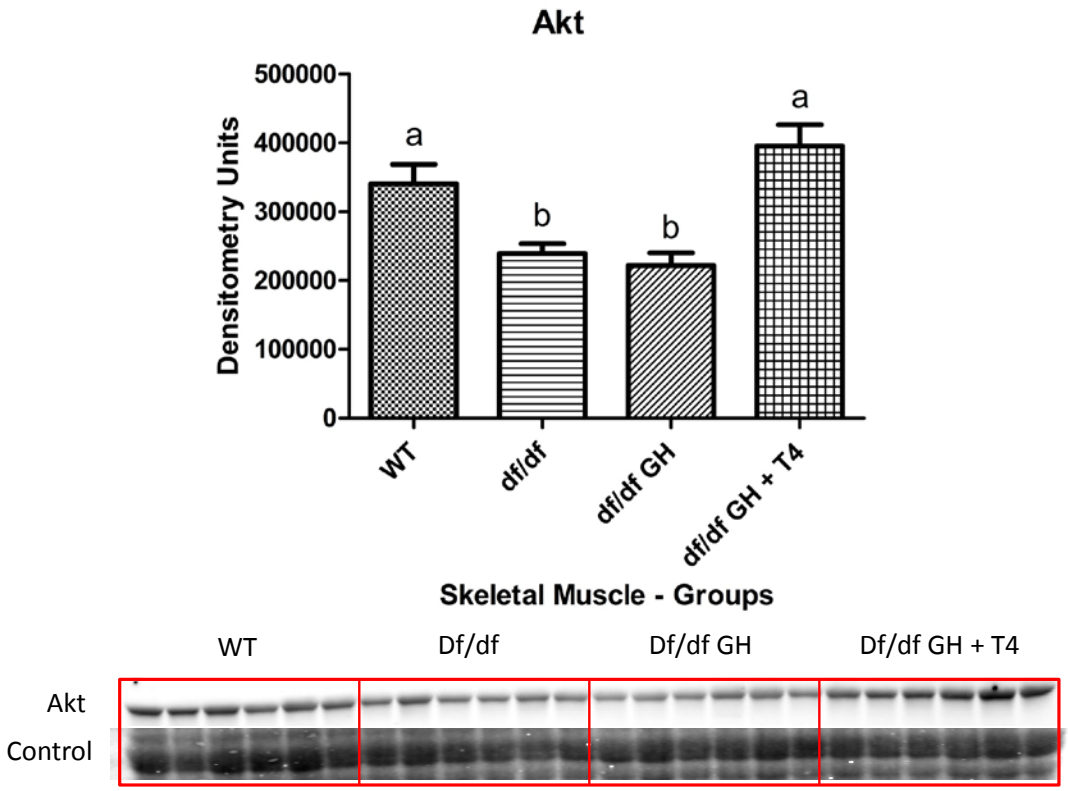


Figure 8 – Expression of total Akt protein in the skeletal muscle of female wild-type mice (WT), Ames dwarf mice (df/df), Ames dwarf mice with GH treatment (df/df GH), and Ames dwarf mice with GH and T4 treatment (df/df GH + T4). WT, n = 6; df/df, n = 6; df/df GH, n = 6; df/df GH + T4, n = 6. Groups that do not share a superscript are significantly different ($p < 0.05$).

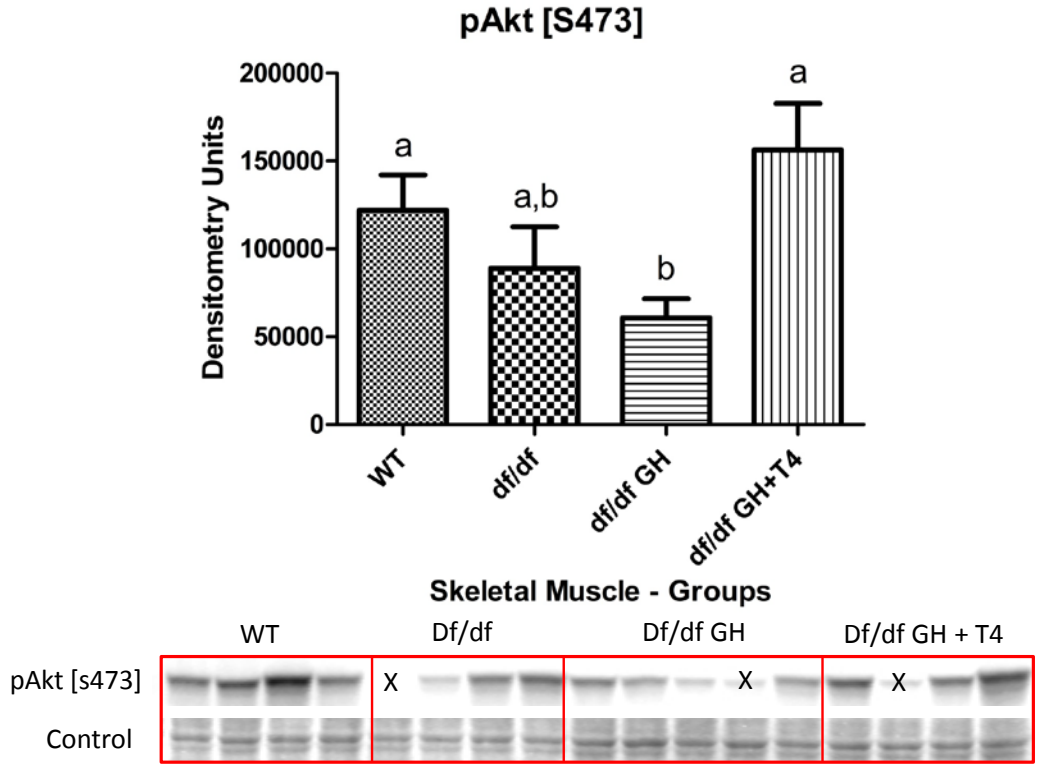


Figure 9 – Expression of Akt protein phosphorylated at serine residue 473 in the skeletal muscle of female wild-type mice (WT), Ames dwarf mice (df/df), Ames dwarf mice with GH treatment (df/df GH), and Ames dwarf mice with GH and T4 treatment (df/df GH + T4). WT, n = 4; df/df, n = 3; df/df GH, n = 4; df/df GH + T4, n = 3. "X" indicates a sample omitted due to unusually low values (probably attributed to incomplete insulin stimulation). Groups that do not share a superscript are significantly different ($p < 0.05$).

DISCUSSION

Due to underdeveloped anterior pituitary glands, Ames dwarf mice are lacking the hormones: growth hormone (GH), thyroid stimulating hormone (TSH), and prolactin. These mice feature several noteworthy characteristics compared to age matched normal control mice, including: smaller size and bodyweight, delayed development and aging with increased lifespan, and enhanced insulin sensitivity and glucose tolerance. Other long-living mutant mouse models, namely the Snell dwarf mouse and growth hormone receptor knock-out (GHRKO) mouse, also share a disruption in the GH / IGF-1 pathway. This suggests that interfering with GH / IGF-1 signaling and increased insulin sensitivity with reduced insulin levels promotes a lifespan enhancing effect [31-34].

In previous studies, reintroducing GH to male Ames dwarf mice via hormone replacement therapy caused the mutants to nearly match the phenotype of normal controls. GH treatment to young mice increases size and bodyweight significantly. Compared to untreated dwarfs, the male dwarf mice on GH therapy showed increased levels of serum glucose, insulin, and IGF-1 along with a decrease in insulin sensitivity and lifespan [26]. GH and T4 combination treatment has also shown significant increases in size and bodyweight of male Ames dwarfs [29]. On the other hand, Ames dwarf mice treated with only T4 showed a bodyweight increase without any significant effects on lifespan [30]. Because of this, GH was initially of greater interest in this study.

In our experiment involving female Ames dwarf mice, both GH treatment and GH + T4 combination treatment significantly increased the dwarf mouse bodyweight to match wild-type

controls. However, combining GH and T4 treatment increased bodyweight to the same extent as GH treatment alone. In other words, adding T4 treatment to the HRT regimen didn't increase bodyweight any greater than GH treatment alone. Also in our study, both the untreated and GH + T4 treated female dwarf mice had no significant difference in fasting blood glucose compared to normal mice. Yet, GH treatment increased dwarf fasting blood glucose relative to normal mice, demonstrating a diabetogenic effect of GH treatment. It seems that thyroxine injections in conjunction with GH treatment helped prevent this elevation in blood glucose. Surprisingly, the untreated Ames dwarf mice had the same blood glucose levels as normal mice instead of the expected lower blood glucose levels.

ITT can be used to measure an animal's whole body insulin sensitivity. After insulin injection, more insulin responsive animals will show a more rapid and pronounced drop in blood glucose levels from baseline. This demonstrates that the same dose of injected insulin produces a greater effect on the body's blood glucose levels, implying better whole-body insulin sensitivity. GTT is used to measure an animal's glucose tolerance. Injection of glucose solution imitates the post-prandial elevation in blood glucose. Animals with greater glucose tolerance will exhibit a more rapid decline in blood glucose to basal levels. This demonstrates the animals' ability to regulate blood glucose levels by detecting and responding to changes in blood glucose, secreting adequate insulin, and responding to the insulin.

In previous experiments with males, GH treatment decreased glucose tolerance and insulin tolerance of Ames dwarf mice, bringing them to levels observed in wild-type mice [23, 26]. Interestingly, regardless of complete normalization of bodyweight, the same dosage of GH

treatment for the same duration didn't significantly affect insulin sensitivity and glucose tolerance in female Ames dwarf mice, as shown by ITT and GTT (Figure 3 and Figure 4). This difference in response to GH treatment may suggest that twice-daily GH injections more closely emulate the natural pulsatile GH secretion pattern of males. This offers a potential explanation for the lack of significant effects on insulin and glucose metabolism in female Ames dwarf mice after GH treatment.

As shown by Dr. Dominici and colleagues, the insulin sensitivity responses of Ames dwarf mice may be tissue specific. According to that study, Ames dwarf skeletal muscle shows a decreased response to large dose insulin stimulation (10Ug/kg bodyweight), which is opposite of their findings with liver. They demonstrated with western blots that when compared to normal mice, Ames dwarf mice skeletal muscle tissue had decreased insulin response or protein phosphorylation of IR, IRS-1, PI3-K, and Akt. In this same study, there were no differences in amounts of IR and Akt protein yet decreased amount of IRS-1, and PI3-K. It was suggested that the decreased insulin sensitivity of the skeletal muscle is a protective mechanism against severe hypoglycemia [21]. This makes sense, considering that skeletal muscle utilizes a large proportion of available blood glucose, having highly insulin sensitive skeletal muscle may deplete the blood glucose to dangerously low levels. In our own analysis, IR, IRS-1, PI3-K, and Akt mRNA also indicated no difference between dwarf and normal mice at the gene expression level in skeletal muscle. However, in the study of male Ames mouse liver after GH and T4 treatment, they found decreased dwarf mRNA to match normal mouse levels for: IR, IRS-1, PPAR-γ, and PGC-1α. At the same time, IGF-1 gene expression of dwarf mice increased to match normal mice due to the dual hormone treatment [29].

In accordance with the Dominici study in 2003, this project showed an increase in IGF-1 and a decrease in GLUT4 gene expression for untreated dwarf skeletal muscle, correlating to a decrease in insulin signaling in skeletal muscle. However, in contrast to the findings in the liver of GH and T4 treated Ames dwarf mice [29], our study showed no effects on gene expression in skeletal muscle to correlate with the ITT and GTT data. This important finding shows that females respond to GH treatment similarly to males for body growth, however, treatment of these GH deficient mice doesn't affect insulin signaling in the skeletal muscle of these long living rodents.

Surprisingly, most of the significant differences in mRNA levels were demonstrated by the GH + T4 treated dwarf mouse group. When referring to Figures 5, 6, and 7 for Akt2, PPAR-γ, GHR, IGF-1, STAT-1, and GLUT4 mRNA, it can be observed that the addition of T4 treatment opposes the actions of GH treatment alone.

On the other hand, some of the data related to an increase of insulin sensitivity. For example, the GH treatment alone increased dwarf IRS-1 and GLUT4 expression levels. GH and T4 combination treatment featured lower gene expression for GH signaling by having decreased GHR, IGF-1, STAT-1, and STAT-5b levels compared dwarf mice treated with only GH.

In terms of protein analysis, the results for total Akt differ than previously shown. The data in this study (Figure 8) shows dwarf mice having less total Akt than normal mice and this is only corrected with combining T4 with GH treatment. However, a previous study shows female Ames dwarf mice having the same levels of total Akt as normal mice, the only difference found in Akt phosphorylation or activation [21]. Furthermore, while GH treatment suppresses Akt

phosphorylation at serine 473 in dwarf skeletal muscle, GH + T4 treatment reverses this change by increasing activation to normal mouse levels. This shows an opposite trend in the liver, where GH treatment of dwarf mice increased ser473 phosphorylation of Akt above normal and dwarf controls [26]. In summary, GH treatment alone didn't increase Akt expression or activation in dwarf skeletal muscle, but GH + T4 treatment did cause increases. Once again, only when T4 was used in conjunction with GH therapy did we find significant changes.

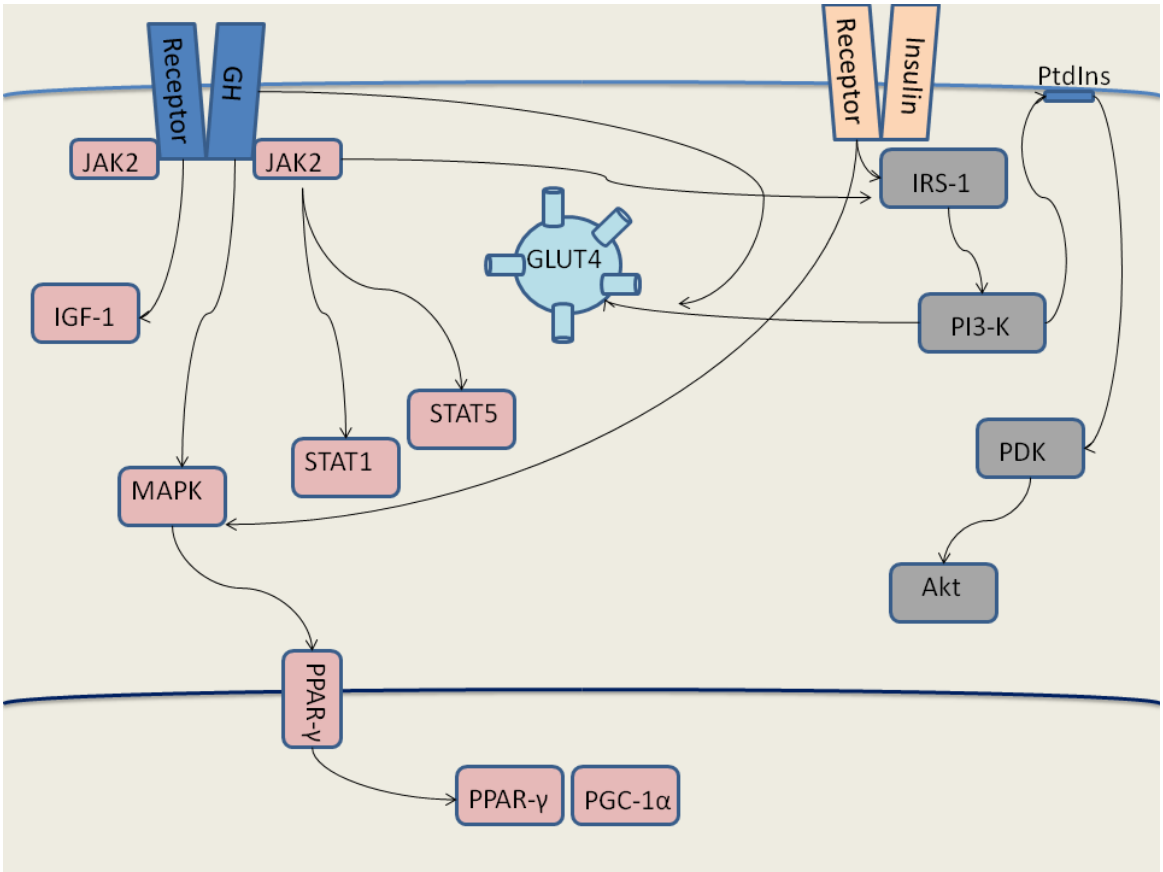
One very large possible confounder for the differences in expression is the fact that we're comparing our female mouse tissue samples with some data from males. In males, GH is present at low basal levels; every few hours they have pulsatile secretion of high amounts of GH and then return to basal levels. Females' basal level of GH is higher in comparison, yet they have pulses smaller in amplitude with greater frequency. Based on these physiological differences in regulation of GH, the twice daily GH treatment regimen for the dwarf mice more closely matches the natural GH secretion patterns of males than females. In addition, this is only half the story with gene expression; changes in the mRNA levels of genes don't always correlate to changes in translation and protein expression. Further experiments and studies must evaluate the levels and activation/deactivation of different signaling proteins to determine what steps of the pathway are being regulated by these hormones in skeletal muscle.

In conclusion, this study found that GH therapy didn't have major effects on insulin signaling in skeletal muscle of female Ames dwarf mice. However, combining T4 treatment with GH therapy suppressed mRNA expression for several genes compared to GH injections alone. T4 seemed to oppose growth hormone's changes in expression of some genes, especially GLUT4

and IGF-1. When looking at the GH + T4 treatment group, thyroxine appeared to be negatively regulating the gene expression of players in GH signaling in skeletal muscle, including the GHR gene. We can note the correlating decrease in IGF-1 gene expression with the addition of T4 to the hormone replacement therapy. We suggest that T4 may be suppressing the gene expression of the GH signaling pathway, therefore counteracting some of the effects of GH replacement in the GH + T4 group. By testing the effects of GH and T4 replacement therapy on skeletal muscle tissue's insulin signaling pathway of anterior pituitary hormone deficient mice, we can help elucidate the role that GH plays in whole body insulin sensitivity, as well as some effects of combining T4 with GH therapy. This in turn may contribute to better understanding of the interactions between these hormones and support development of new, safe ways to increase insulin sensitivity in Type 2 Diabetes patients and the elderly, promoting better health and quality of life for the future.

APPENDIX

Basic GH/Insulin Pathway Investigated



Supplement 1 - Simplified flow chart of the Growth Hormone (GH) and Insulin Signaling pathway, demonstrating most of the proteins investigated in this study. Adapted from Dominici et al., 2005 [24].

Agarose Gel Electrophoresis

Mixed 260mL TAE (1x) + 6.25g agarose powder in beaker and boiled in microwave. Added 25µL SYBR Gold and mixed well before pouring into casting tray with comb. Gel cooled to room temperature and solidified in approximately 30 min. After solidifying, gel was placed into buffer bath. 5µL of sample was mixed with loading dye on parafilm and then loaded into wells. Electrophoresis was run for 40 min with the following specs: 120V / 3A / 300W. Gel was visualized on Kodak Imaging device with Carestream program. Epoch Plate Reader with Gen5 software was also used to quantify RNA concentration using set programming.

Nano-drop Protocol

2µL of RNA sample was placed in each well of the Take 3 plate for the Epoch Gen 5 Plate Reader, along with duplicates of RNase free water for blanks. The absorbance was read at 260nm to determine relative RNA concentration and an absorbance ratio of 260/280 greater than 1.9 indicated sufficient purity.

RT-PCR Protocol

This project made use of the Applied Biosystems 7500 Fast Real-Time PCR System.
Instrument: 7500 Fast (96 wells)
Type of experiment: Quantitation – Comparative C_T ($\Delta\Delta C_T$)
Reagents: SYBR Green (Including Melt Curve)

Speed: Fast (~40 min)

45 cycles

Used Applied Biosystems Protocol except Anneal/Extend Temp was changed to 64°C.

Enzyme Activation: 45°C for 20 seconds

Denature: 95°C for 3 seconds

Anneal/Extend: 64°C for 30 seconds

RT-PCR Primers

Ordered from Integrated DNA Technologies and designed by Xu Zhi

Gene	Primer	Sequence (5' → 3')
B2M	Forward	AAG TAT ACT CAC GCC ACC CA
	Reverse	CAG GCG TAT GTA TCA GTC TC
IR	Forward	GTT CTT TCC TGC GTG CAT TTC CCA
	Reverse	ATC AGG GTG GCC AGT GTG TCT TTA
IRS-1	Forward	AGC CCA AAA GCC CAG GAG AAT A
	Reverse	TTC CGA GCC AGT CTC TTC TCT A
PI3-K	Forward	TAG CTG CAT TGG AGC TCC TT
	Reverse	TAC GAA CTG TGG GAG CAG AT
Akt2	Forward	GAG GAC CTT CCA TGT AGA CT
	Reverse	CTC AGA TGT GGA AGA GTC AC
GHR	Forward	AGG TCT CAG GTA TGG ATC TTT GTC A
	Reverse	GCC AAG AGT AGC TGG TGT AGC CT
IGF-1	Forward	CTG AGC TGG TGG ATG CTC TT
	Reverse	CAC TCA TCC ACA ATG CCT GT
STAT-1	Forward	CGA CCA GTA CAG CCG CTT TT
	Reverse	CGG GAT CTT CTT GGA AGT TAT CCT
STAT-5a	Forward	ACC ACC TTC AGT GTA AGG AG
	Reverse	AAA GGT CAC CAC CGC TTT AG
PPAR-γ	Forward	GTC AGT ACT GTC GGT TTC AG
	Reverse	CAG ATC AGC AGA CTC TGG GT
PGC-1α	Forward	TAC GCA GGT CGA ACG AAA CT
	Reverse	TGC TCT TGG TGG AAG CA
GLUT4	Forward	ATT GGC ATT CTG GTT GCC CA
	Reverse	GGT TCC GGA TGA TGT AGA GGT A

SDS-PAGE

Bio-Rad Running buffer (1X TGS) and Transfer buffer (1X TG) were almost always made fresh before use according to directions listed on the package. 5µL of molecular weight marker loaded in 1st well followed by 14µL of samples; the final empty well was loaded with 14µL of sample buffer (no protein). While the dye was in the stacking gel, the voltage of the power unit was set to 80V (for approximately 40 min). When the proteins/dye reached the resolving gel, the voltage was increased to 100V (for approximately 60 min). Running buffer (1X TGS) was reused once, or until the current approached 0.7A.

Antibodies

Both the Akt and phospho-Akt s473 primary (1°) antibodies were purchased from Cell Signaling Technology and were sourced from rabbits. Antibodies were added to a 1:1 mixture of Odyssey Blocking Buffer and PBS-T in a 1:750 ratio and stored at -20°C until use. When ready to be used, they were quickly thawed in a 37°C water bath and washed the membrane with gentle shaking overnight in 4°C. When finished, the antibody solutions were refrozen at -20°C until re-use. Each lab member prepared their own antibody solutions to prevent cross-contamination.

The secondary (2°) antibody was Peroxidase Conjugated Affinity Purified anti-Rabbit IgG (H&L). It was purchased from Applied Biological Materials Inc. and sourced from goats. It was prepared as a 1:15,000 dilution by adding 2µL of antibodies to a 30mL solution consisting of Odyssey Blocking Buffer and PBS-T in a 1:1 ratio (15mL of each). This was stored at 4°C

until use and protected from light by aluminum foil wrapping. When in use, it washed the membrane with gentle shaking for 1 hour while being protected from light.

Western Transfer and Western Blot

PVDF membrane was first cut to a size that matched the polyacrylamide gel and then activated for 1 min in methanol (MeOH). Transfer buffer was cooled in 4°C prior to use. The wet western transfer was performed with the PVDF membrane and polyacrylamide gel stacked on top of each other and both sandwiched between filter paper and sponges. The gel was on the side of the negative electrode while the membrane was on the side of the positive electrode, ensuring the electrical current would direct the proteins from the gel to the membrane. The sandwich was placed in the running tank, submerged in cooled transfer buffer along with ice pack and a magnetic stirring rod. The transfer was performed at 80V for 45 minutes.

When finished with the transfer, the membrane was rinsed in phosphate buffered saline (PBS) and then blocked in 5% milk (1.5g dry milk powder in 30mL of PBS) for 1 hour. This step was succeeded by: a 20-30 min wash with phosphate buffered saline with tween (PBS-T), 1° antibody incubation, another 20-30 min wash with PBS-T, 2° antibody incubation, and then finished off with a final 20 min PBS-T wash and storage in PBS. The washes in PBS-T were actually 5 minute washes repeated 4-6 times, totaling 20-30 minutes.

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